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14. ABSTRACT
Too many prostate-cancer treatments, especially those relying on the suppression of androgen, eventually fail to slow the advance of the disease. One explanation for this situation is the absence of any systematic knowledge on the role and function of the androgen receptor (AR) in the course of prostate cancer development. Recent findings indicate that the AR is the key master regulator (transcription factor) that determines disease progression to androgen independence, which ultimately contributes to death from prostate cancer. During the third year of this grant funding, we concentrated our efforts on the understanding of how transcriptional control of the AR at target loci is achieved as the PCa cells escape from androgen ablation therapy to become treatment resistant. We found that histone H3 lysine 4 methylation patterns are unique in ablation resistant PCa cells. Furthermore, globally the AR and certain histone modifications co-locate in discreet areas in the nucleus. In the final no-cost extension year of the grant we will complete our work on other AR traget genes. Thus, armed with a deeper knowledge of the hormonal and receptor requirements as well as mechanisms associated with prostate cancer growth and expansion, we may be able to develop therapies that prolong lives. Understanding the behavior of the AR, as documented above, is a first step in that quest.

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Introduction:

Mounting evidence suggests that androgen receptor (AR) signaling is critical in all phases of prostate cancer (PCa) development, including disease initiation, androgen-dependent tumor growth and late stage castrate-resistant disease. Resistance to androgen ablation therapies is not accompanied by a loss of AR, but rather associated with reemergence of androgen signaling and re-expression of AR-regulated genes (Balk, 2002; Buchanan et al., 2001; Debes and Tindall, 2004; Feldman and Feldman, 2001). Although a recent study showed that the general androgen signaling signature was attenuated in ablation-resistant metastatic PCa, the AR target genes with strong androgen promoter-enhancer elements remained active (Tomlins et al., 2007). Ablation-resistant PCa clones were selected and required minimal androgen signaling for continued survival in the castrated state. Three studies exemplify the AR-dependence of ablation-resistant PCa cells. First, disruption of AR expression by a specific antibody or ribozyme inhibited proliferation of ablation-resistant PCa cells in the absence of androgens (Zegarra-Moro et al., 2002). Second, increased AR expression was necessary and sufficient to convert androgen-sensitive PCa to an ablation-resistant state (Chen et al., 2004). Third, specific expression in mouse prostate epithelial cells of an AR transgene, which contained a gain-of-function mutation (with increased basal activity and response to coregulators) resulted in PCa development in 100% of the animals (Han et al., 2005). These lines of evidence prove that aberrant AR signaling is sufficient to cause PCa and that under certain conditions the AR may act as an oncogene. Studies funded by this idea development grant are aimed to better understand the underlying mechanisms that govern this activity of the AR.

Body:

Based on the reviewer's feedback from our progress report two years ago (2004-2005), we have reformulated our specific aims to accommodate findings made during the 1st year of funding. The new SOW, submitted in April 2006 and subsequently accepted, is as listed below:

Revised Statement of Work for W81XWH-04-1-0823 (April 2006)

Originally two specific aims were formulated namely:

***Specific Aim #1: To identify non-steroidal pathways modulating AR activity*.**

Specific Aim #2: To elucidate how non-steroidal signaling determines transcription complex compositions and histone code modifications in regulatory areas of AR target genes.

We have essentially completed the first (see progress report October 2005), which led to one research paper (Kim et al., 2005) and one review (Kim and Coetzee, 2004).

We have subsequently concentrated on the second specific aim and have found that androgen-independent expression of PSA in androgen-independent prostate cancer

cells does not rely on the direct occupancy of the AR at the PSA locus, but is nevertheless affected PSA expression indirectly via unknown AR-dependent mechanism(s) that influence the expression from some but not all AR target genes. (Jia and Coetzee, 2005). We will therefore do the following tasks to unravel the mechanisms of this interesting finding:

Task 1: **To analyze AR target gene chromatin architecture in the LNCaP/C4-2B cell culture model of progression to androgen-independence of prostate cancer cells (months 1-12).**

a. **Compare chromatin integrated gene expression with transiently transfected luciferase reporters (months 1-3).**
b. **Measure histone modifications (acetylation & methylation) across the entire PSA locus (months 4-6).**
c. **Check gene expression of PSA and histone modifications at the PSA locus after siRNA knock-down of the AR (month 7-10).**

Task 2: **Develop ChIP and Q-RT PCR assays for other PSA target genes (months 10-15).**

Task 3: **Perform similar analyses as outlines in task 1 on the other AR target genes (months 15-18).**

Task 4: **Perform similar analyses as outlines in task 1 using another cell-culture-independent model of progression to androgen-independence, namely the xenograft CWR22 mouse model from which we already have frozen tissue obtained by funding from another DoD grant, W81XWH-04-1-0049 (months 15-18).**

Task 5**: Write manuscripts (months 12-18).**

Because specific aim #1 has been completed, we have continued to work on specific aim #2 as formulated above and outlined below.

Tasks 1 and 4 are essentially completed and published (Task 5), although aspects of task 1 were further explored due to a recent publication (Heintzman et al., 2007) (see below). Our previous results were summarized in our progress report of last year (October 2006).

We have therefore concentrated on an extension of task 1 and on tasks 2 & 3.

Task 1: Chromatin Architecture at AR target sites:

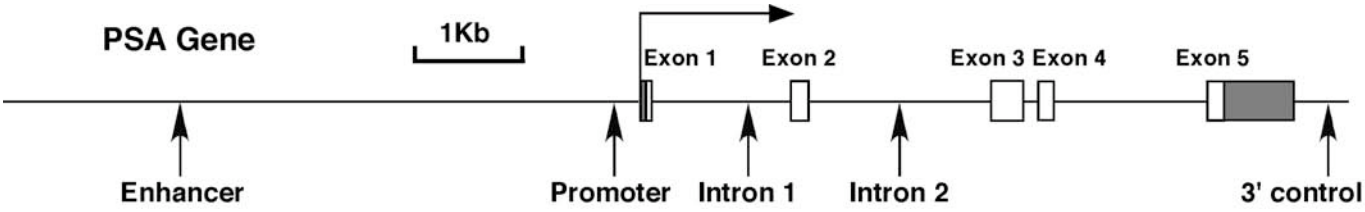
In a recent *Nature Genetics* article entitled 'Distinct and Predictive Chromatin Signatures of Transcriptional Promoters and Enhancers in the Human Genome', Heintzman *et al* proposed that histone H3 lysine 4 (H3K4) methylation may be used to

distinguish enhancers from promoters; enhancers were preferentially mono-methylated, whereas promoters were preferentially tri-methylated. Although this is an interesting and novel concept, and probably applies to many promoters and enhancers, exceptions exist. We have now recorded mono-, di- and tri-methylation levels of histone H3K4 at several sites in the PSA locus during PCa conversion to ablation resistance and made several interesting observations.

We analyzed histone modifications at the PSA locus in androgen-dependent LNCaP and its ablation-resistant derivative C4-2B cell line. Levels of mono-, di-, and tri-methylation of histone H3K4 at the enhancer, the promoter and gene body (intron 1 & 2) in the two cell lines were analyzed in relation to the expression of PSA, as modulated by dihydrotestosterone (DHT) (Table). [Note: The relative modified methylation levels between mono-, di- and tri-methylation cannot be compared, since the efficiency of the antibodies used to precipitate them may not be the same]. All three types of H3K4 methylation levels were detected at the enhancer and promoter in both cell lines regardless of DHT stimulation. Di- and tri-methylation levels (but not mono-methylation levels) in the gene body increased up to 3-fold in C4-2B vs. LNCaP cells regardless of short-term hormone treatment. Such modified chromatin regions (domains), as found in the gene body, may provide a robust epigenetic memory to maintain expression of genes in a lineage-specific manner regardless of short-term changes. Their relatively large size across linear DNA ensures that each daughter chromosome inherits a significant proportion of the modified histones, which in turn promote similar modifications of the newly assembled neighboring histones. We conclude that chromatin domains are epigenetically selected during androgen-dependent to castrate-resistant conversion of PCa cells, resulting in efficient AR-mediated gene expression and cell growth. In contrast to the prediction (Heintzman et al., 2007), H3K4 methylation may not distinguish all enhancers from promoters, but does seem to mark lineage-specifically the gene body of PSA. Such signatures across chromatin domains may additionally indicate epigenetic, and thus inherited marks, for efficient transcription.

In summary, we made two main findings: First, no dramatic differences in H3K4 methylation levels were observed that unambiguously distinguished the PSA enhancer from the promoter. Second, significantly elevated H3K4 di- and tri-methylation levels were evident in the PSA gene body and were related to PCa progression to ablation-resistance and not in the short term in response to increased PSA expression brought about by hormone treatments. The data add additional insight into the work of Heintzman *et al* (Heintzman et al., 2007) by extending it to histone H3K4 epigenetic/inherited modifications at an important locus in PCa.

Table: Histone H3K4 methylation levels at the PSA locus



				ChIP (value/input)		
Cell type	***±DHT***	***PSA Expression***	***Site***	***H3K4 Mono***	***H3K4 Di***	***H3K4 Tri***
LNCaP	-	-	Enhancer	6.4 ± 0.8	4.5 ± 0.6	2.5 ± 0.6
			Promoter	6.5 ± 0.5	4.1 ± 0.3	2.6 ± 0.3
			Intron 1	4.9 ± 0.8	3.2 ± 0.2	2.1 ± 0.3
			Intron 2	5.6 ± 0.5	3.2 ± 0.2	2.4 ± 0.9
			3' control	3.1 ± 2.0	1.2 ± 0.8	1.0 ± 0.7
	+	+++	Enhancer	4.1 ± 0.7	2.5 ± 0.1	1.7 ± 0.1
			Promoter	6.1 ± 0.3	3.4 ± 0.2	2.4 ± 0.2
			Intron 1	4.7 ± 0.7	3.6 ± 0.8 *	2.5 ± 0.3 *
			Intron 2	4.8 ± 0.2	3.6 ± 0.6	3.1 ± 0.7
			3' control	3.4 ± 0.9	1.5 ± 0.3	1.2 ± 0.1
C4-2B	-	+++++	Enhancer	4.1 ± 0.2	3.4 ± 0.1	1.6 ± 0.1
			Promoter	5.2 ± 0.3	6.2 ± 0.7	3.7 ± 0.7
			Intron 1	5.5 ± 0.4	**9.2 ± 0.7** *	**6.0 ± 0.4** *
			Intron 2	5.3 ± 0.8	**8.1 ± 1.6**	**5.0 ± 0.7**
			3' control	3.5 ± 0.4	2.4 ± 0.4	1.3 ± 0.1
	+	++++++++ ++++++++	Enhancer	3.4 ± 0.1	1.9 ± 0.3	0.9 ± 0.1
			Promoter	3.2 ± 0.6	3.3 ± 0.4	2.0 ± 0.2
			Intron 1	4.2 ± 0.1	**9.2 ± 1.1**	**6.5 ± 0.6**
			Intron 2	3.9 ± 1.3	**8.1 ± 1.9**	**5.8 ± 1.6**
			3' control	3.1 ± 0.6	1.7 ± 0.6	1.0 ± 0.4

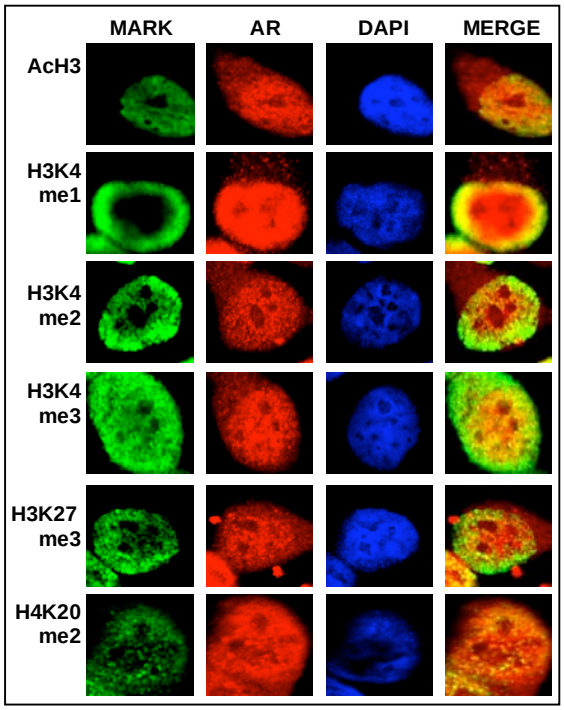
**LNCaP vs. corresponding sites in C4-2B, two-sided p<0.006*

Task 1: Double immunofluorescence of AR and histone modification marks in C4-2B cells

We tested whether histone modification marks spatially co-exist with the AR in the nucleus of C4-2B cells. As shown by immunofluorescence (Figure 7) AcH3, H3K4me2, H3K4me3, H3K27me3, and H4K20me2 were diffused throughout the nucleus. In contrast, H3K4me1 appeared to be enriched toward the nuclear periphery. While the significance of the unique nuclear sublocalization of H3K4me1 is interesting

(Kosak and Groudine, 2004), the important point is that all histone modification marks tested partially co-localized with the AR.

Figure 1. Global localization of AR and histones with specific modification. DHT-treated C4-2B cells were probed with antibodies against the AR and the histone modification marks listed to the left. Co-localization of the AR (red) and sites with each histone modification (green) are demonstrated in the merged images.



Task 2 & 3: Assesment of novel AR target genes

We have started this work but at present it is not complete. Therefore we have asked, and were awarded, a no-cost extension until next year. We will report on our progress then in the final report.

Key Research Accomplishments during the past year:

1. Increased AR signaling is a consequence of chromatin modifications at particular loci, contributing to the transition of PCa cells to ablation resistance.
2. The chromatin-modified state referred to above depends on sustained AR activity.
3. Histone H3 lysine 4 methylation patterns are unique in ablation resistant PCa cells.
4. Globally the AR and certain histone modifications co-locate in discreet areas in the nucleus.

Reportable Outcomes:

Jia, L, Shen, HC,Wantroba, M, Khalid, M, Liang, G, Wang, Q, Gentzschein, E, Pinski, JK, Stanczyk, FZ, Jones, PA and **Coetzee, GA**: Locus-wide chromatin remodeling and enhanced androgen receptor-mediated transcription in recurrent prostate tumor cells. Mol. Cell. Biol. 26:7331-7341, 2006.

Jia, L and **Coetzee, GA**: BROAD CHROMATIN MARKINGS INDUCE SUPER-EFFICIENT TRANSCRIPTION IN CASTRATE-RESISTANT PROSTATE CANCER
DoD Impact meeting, Atlanta, September 2007.

Conclusions:

Overall and final conclusions are pending.

Appendices:

None

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